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SUMMARY

We introduce the concept of 'silent learning' - the capacity to learn despite neuronal cell-firing being largely absent. This idea emerged from thinking about dendritic computation[1, 2] and examining whether the encoding, expression and retrieval of hippocampal-dependent memory could be dissociated using the intra-hippocampal infusion of pharmacological compounds. We observed that very modest enhancement of GABAergic inhibition with low-dose muscimol blocked both cell-firing and the retrieval of an already formed memory, but left induction of long-term potentiation (LTP) and new spatial memory encoding intact (silent learning). In contrast, blockade of hippocampal NMDA receptors by intrahippocampal D-AP5 impaired both the induction of LTP and encoding, but had no effect on memory retrieval. Blockade of AMPA receptors by CNQX impaired excitatory synaptic transmission and cell-firing, and both memory encoding and retrieval. Thus, in keeping with the synaptic plasticity and memory hypothesis [3], the hippocampal network can mediate new memory encoding when LTP induction is intact even under conditions in which somatic cell-firing is blocked.

RESULTS

Encoding and retrieval of declarative memory are the two sides of a coin with respect to the neural mechanisms of learning and memory. Encoding refers to the acquisition of new information, whereas retrieval involves the reactivation of previously learned memory traces. Identifying the neural activity associated with specific memory processes such as these is a necessary step to understand of how information-processing circuits operate. The present study tests whether (a) memory retrieval requires cell-firing, enabling information transfer within and between networks; and (b) memory encoding may minimally require the induction and expression of synaptic plasticity, with little or no somatic cell-firing. The occurrence of learning would not be observable, but we argue that recent advances in the physiology of dendritic computation predict such 'silent learning' could occur.

Loss-of-function manipulations such as lesions, drugs and molecular interventions have long been deployed to look for 'learning impairments' in specific tasks. Many studies focus on misleading learning curves during an intervention[4, 5] that likely reflect impacts on encoding, storage, consolidation and/or retrieval without dissociating their relative contributions. Lesions can also cause 'performance' effects (i.e. deleterious effects upon sensorimotor processes or motivation). Specific protocols to dissociate encoding and retrieval definitively include: for *retrieval* - monitoring performance on the first trial of new training *before* any new learning can take place[6]; and for *encoding* - application of the intervention *during* the new training, but testing *later* in its *absence* when retrieval should be operating normally[5]. A suitable protocol for the watermaze is the delayed-matching to place (DMP) or 'everyday memory' procedure which involves learning a new daily spatial location of the escape platform during each session[7]. The principle is that, on each session, the animals first retrieve a memory of where escape was possible during the *previous* session, and then update their memory by encoding where the escape platform is now located during the *current* session. The effectiveness of this memory encoding is tested on a *subsequent* session in which the animals should again demonstrate memory of the preceding session.

The main study we examined the impact of drugs over 5 successive series of 3 linked sessions (hereafter called s1, s2 and s3) in a within-subject manner (Figure 1A). It followed animal handling, bilateral drug cannula implantation and initial training over 8–10 sessions, during which the 16 animals learned the DMP task well each day with a new platform location chosen for each session of 4 learning trials (Figures 1A,B,E). An animal might be trained to encode that the escape platform is in the NW (North-West) quadrant on all 4 trials of session 1 (s1, Figure 1B). On the next session (s2), using an Atlantis Platform procedure [8] in which memory retrieval is assessed during the first 60 s of the first trial before any new learning takes place (Figures 1B,C,D and S1), the animal should remember this NW location (red dotted circle) by searching there (during the first 60 s) before learning that the hidden platform had been moved to SSW (South-South-West). Encoding of this new location occurs during the four escape trials of s2, updating and over-writing the memory acquired in s1. The platform is moved again for s3, again allowing an analogous test of memory during the first 60 s. In this protocol, *memory retrieval* is procedurally dissociated from new *memory encoding*.

This procedure enabled effective memory encoding of each daily location, with performance typically characterised by a stable monotonic decline in first-crossing latency across trials within each session (Figure 1E; Data S1). The swim-latency on trial 1 (≃50-70 sec) was stable across sessions, as was the 'savings' in latency of approximately 30 s between trials 1 and 2 of each session. Asymptotic performance over trials 2-4 reflects the effectiveness of memory updating.

In vivo Hippocampal Physiology

Previous studies have established that blocking hippocampal N-methyl-D-aspartate (NMDA) receptors, via pharmacological [D-2-amino-5-phosphonopentanoate (D-AP5)] or molecular-genetic interventions, limits memory encoding without effect on retrieval[7, 9-12]. This is observed with 1-2μl bilateral infusions into dorsal hippocampus, with autoradiography indicating substantial spread along the longitudinal axis following a 2μl infusion[13]. This behavioural pattern is mechanistically linked to blockade of NMDA receptor-mediated activity-dependent synaptic plasticity[3, 14]. Blocking α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors pharmacologically limits encoding, consolidation and retrieval [15]. Prior focus on AMPA and NMDA receptors left uninvestigated the possible contribution of γ-aminobutyric acid (GABA-ergic) inhibition which is known to regulate long-term potentiation (LTP) induction[14]. The importance of dynamic patterns of inhibitory activity is now recognised as functionally important[16, 17], along with learning-associated changes in inhibitory circuitry that can affect the fidelity of memory[18, 19].

Using *in vivo* electrophysiology in male Lister Hooded rats (n=20) to identify appropriate drug concentrations using and the time course of their effects (Figure 2), we sought drug doses that would differentially affect (a) cell-firing, (b) fast synaptic transmission, and/or (c) activity-dependent synaptic plasticity *in vivo* in the hippocampal formation. We chose to monitor the dentate gyrus electrophysiologically, while recognising that an infusion targeting the outer molecular of the dorsal dentate gyrus would diffuse throughout dorsal CA1 and CA3 as well. In the case of muscimol, it should incur reasonably widespread binding to somatic and dendritically located $GABA_A$ receptors.

A key new finding is that low-dose muscimol blocked cell-firing but not LTP induction. Infusion of 0.38 nanomoles of muscimol caused a modest 35% decrease of the field excitatory postsynaptic potential (fEPSP) (Figures 2A,B; Data S2), but the dentate population spike ceased almost completely from 30 min post-infusion for 2 hr (measured at 1.0 to 1.5 mm from the infusion cannula; Figures 2C,D). Strikingly, low-dose of muscimol infusion left induction of LTP intact (Figures 2E,F; Data S2). Enhanced GABAergic inhibition normally blocks the induction of LTP[14], but our dose titration down to 0.38 nanomoles achieved a situation in which LTP induction was intact despite the absence of pre-induction cell-firing. This low dose of muscimol did not prevent a very small population spike post-LTP (Figure S3; <3 mV), a change that is unlikely to be relevant to the more distributed patterns of learning-associated dendritic and somatic neural activity in the freely-moving animal (see below).

In contrast, D-AP5 (60 nanomoles) caused a transitory disruption of the fEPSP before a return to baseline within 30 min, and a partial albeit more sustained inhibition of the population spike (Figures 2A–D; Data S2). However, as expected, it blocked LTP induction (Figures 2E,F; Data S2). CNQX (6 nanomoles) caused the fEPSP to be completely inhibited within 30 min with respect to both synaptic activation and cell-firing (>90% decrease for over 1 hr, Figures 2A–D). In the absence of a measureable fEPSP, CNQX was not tested with respect to LTP.

Silent Learning

The stage was now set to conduct the companion behavioural study using male Lister Hooded rats (n=16) trained in the task and now subject to intrahippocampal drug infusions. Each session was conducted 'blind' with respect to drug-assignment[7, 20], using a fully counterbalanced repeated-measures within-subjects design, such that each animal served as its own control across successive 'linked' sessions, consisting of a pre-drug session (s1), drug session (s2) and post-drug session (s3) within each block (Figure 1A).

Our second key finding was 'silent learning' with low-dose muscimol. Representative swim-paths show Rat-G7207 treated with muscimol searching

appropriately on s1, but swimming all over the pool on s2 without memory of the s1 platform location until it eventually found the new escape location after >60 s; on the following drug-free s3, this rat searched successfully in a focused zone around the s2 location (Figure 3A, panel with pool shaded blue, note multiple crossings of the s2 location on s3). This pattern of searching behaviour implies that memory encoding was intact during s2 under muscimol, despite memory expression being blocked. Representative paths of other rats show respectively: good memory for each previous session for aCSF; good memory retrieval but no new learning under D-AP5; and no memory retrieval or new learning under CNQX. Quantitatively, we observed a double dissociation between the impact of the three drugs on memory encoding and retrieval (Figure 3B; two-way ANOVA: significant Drug × Sessions interaction: $F_{6,90} = 3.65$, $p = 0.003$). This statistical interaction justified separate analyses of each drug condition compared to aCSF vehicle, as well as planned comparisons to chance-level performance.

Vehicle sessions (aCSF) showed good above chance memory (chance = 4%, dotted line) across all three sessions (Figure 3B, black bars; Data S3). With low-dose muscimol, the animals were at chance on s2 with the animals failing to remember the previous session, but above chance for the location trained under the drug on s2 when tested during trial 1 of s3 (note U-shaped function in Figure 3B; Data S3). That is, low-dose muscimol was permissive for new encoding despite causing a complete block of memory retrieval. The opposite pattern prevailed with D-AP5, with above chance retrieval of s1 on trial 1 of s2, but chance performance during trial 1 of s3. With CNQX, the animals' memory of s1 displayed on trial 1 of s2 and their memory of s2 on trial 1 of s3 were both at chance. Thus, CNQX treated rats could neither retrieve nor encode.

In this protocol, the animals 'update' their memory during each session - akin to the concept of 'headed records' in which human subjects often remember the last thing that happened but tend to overwrite earlier events[21]. Updating should only be observed if new learning occurs on s2; thus, a distinct pattern of drug effects is expected on s3. The specific prediction is that D-AP5 and CNQX would block memory updating, whereas aCSF and low-dose muscimol would both be permissive of memory overwriting. We quantified the swim search pattern on s3 with respect to the proportion of time spent in the correct zone for s1 (i.e. *2 sessions back;* Figures 3C,D) and compared these values

to those for s2 (*1 session back*). The overall ANOVA was thus a 2 × 4 analysis for the time spent searching during s3 in the locations used on s1 and on s2 as a function of the 4 drugs, revealing a highly significant Session-Memory × Drug interaction ($F_{3,45} = 7.35$, $p = 0.001$). Unpacking this triple interaction using planned comparisons revealed that the average level of memory measured on s3 after CNQX or D-AP5 during s2 was higher for the s1 location than for s2 (i.e. minimal updating); whereas, with aCSF and muscimol, the opposite pattern prevailed. The orthogonal comparison for this contrast was significant, but a graphically simpler analysis is to look at the absolute level of memory for the s1 location that is expressed during s3 (Figure 3C). The ANOVA for these data also showed a significant drug effect ($F_{3,45} = 3.64$, $p < 0.05$). As predicted, memory for the s1 location after aCSF or muscimol had been infused on s2 was lower than when D-AP5 or CNQX were infused ($F_{1,45} = 8.65$, $p < 0.01$; Figure 3C). This successful updating under muscimol on s2 argues against the retrieval deficit displayed under the drug on s2 being a mere 'performance', 'off-target' or 'state-dependent' effect, as it is unclear how such effects could *selectively* affect retrieval but not memory encoding. Illustrative paths are shown following the administration of aCSF (successful updating) and CNQX (no updating) on different s2 sessions (Figure 3D).

A concern was that intact memory encoding with low-dose muscimol is some artefact of differential spread of the drug, the most likely possibility being that the infusion was restricted to a *small* region of the dorsal HPC. This might have been sufficient to disrupt cell-firing during retrieval and pattern completion, but insufficient to affect new learning within a larger volume of *unaffected* dorsal and intermediate hippocampus. The problem with this interpretation is that the diffusion of D-AP5 and CNQX is likely to have been similar to that of muscimol (MWs = 197, 232 and 114 respectively), and thus new encoding should also have occurred for these drugs - which it did not. This suggests that our deliberate choice of a 2 µl infusion volume achieved substantial spread along the longitudinal axis. Anticipating this, we conducted additional electrophysiological studies with recording in the intermediate zone of the longitudinal axis of hippocampus following infusion of muscimol into the dorsal/septal region (infusion 2.5 mm from the recording electrode, Figure S2B). Inhibition of the fEPSP (Figures S2C,E, circa 23%)) was slightly less than at the more proximal recording site (35%) but, importantly, cell-firing remained almost completely blocked (circa 86%, Figures S2D,E). We also attempted to look at drug

diffusion directly using fluorescent muscimol [22]. The analysis of 3 animals subject to bilateral 2 µl infusions of 0.19 mM fluorescent muscimol bodipy, indicated diffusion along the longitudinal axis of up to 3.5 mm (Figure S2F,G), with minimal spread into retrosplenial cortex (RSC) or overlying parietal cortex. But this measure is conservative as the molecular weight of fluorescent muscimol (MW = 607) is much larger than that of muscimol itself (MW = 114), and it is more lipophilic by virtue of the fluorescent label.

A third but unlikely possibility is that drug diffusion reaches beyond hippocampus to retrosplenial cortex, long implicated in spatial memory[23]. On this view, muscimol in RSC may be contributing to the impaired memory retrieval. We had hoped that data on diffusion of fluorescent muscimol could definitively address this issue, but it is unclear how this account would could explain effective new memory encoding in RSC. Extensive diffusion of muscimol itself is surely unlikely as the closely packed, myelinated fibres of the overlying alveus and corpus callosum would restrict this from happening. As in our earlier autoradiographic and regional cerebral blood flow studies of glutamate receptor antagonists [15, 24], there were non-spherical 'rugby-ball' shaped diffusion volumes within hippocampus, also observed with fluorescent muscimol [22]. Some disruption to cell-firing in RSC might nonetheless contribute to the retrieval deficit seen with muscimol and CNQX, possibly by affecting the translation of memory representations from allocentric to egocentric to enable accurate heading to the remembered escape location.

DISCUSSION

The present findings point to a new concept which we shall refer to as 'silent learning' - new memory encoding in the absence of cell-firing. Silent learning corresponds behaviourally to new episodic-like memory encoding in the absence of memory retrieval. We suggest that this can sometimes occur if LTP induction is intact during cellular silence, allowing activity-dependent synaptic potentiation to encode a new spatial memory as a distributed pattern of potentiated synapses in the hippocampus (dentate, CA3 and/or CA1). Cell-firing would not always be necessary.

From a behavioural perspective, the concept of 'silent learning' is distinct from the classical concept of 'latent learning' which refers to successful learning in the absence of reward [25]. Latent learning was a challenge for Hull's drive reduction theory[26] which

supposed that animals had to be motivated to learn ('drive') and that stimulus-response connections were 'stamped-in' by 'drive-reduction' following reward. Silent learning is different, being more consonant with Tolman's 'cognitive map' theory[25] which asserted that learning could occur in the absence of reward, resurrected in O'Keefe and Nadel's theory of hippocampal function[27].

The distributed associative neural circuit of the hippocampus[28] has been proposed to operate in distinct encoding and retrieval modes at different phases of the theta rhythm[29]. We reasoned that it might be possible to realise a double dissociation of the processes of memory encoding and retrieval as a function of task demands. Blocking NMDA receptor activation is permissive for memory retrieval but prevents encoding[9, 12], whereas AMPA receptor inhibition blocks both[30]. Our new finding indicates that, even though memory retrieval fails to occur when cell-firing in the hippocampus is blocked by low-dose muscimol, new encoding can occur provided synaptic plasticity is intact and fast synaptic transmission only modestly affected. Although not accompanied by electrophysiological analysis, a previous behavioural pharmacology study was also suggestive of such a possibility, with spared 'extinction learning' being observed in an inhibitory avoidance task during intrahippocampal low-dose muscimol (approximately twice as high as we used[31]). Impaired retrieval has been shown to occur with higher doses of muscimol[32], but the possibility of intact or impaired learning was not investigated.

The qualification is the possibility of acute 'off-target' alteration of neural circuits (e.g. RSC) that were not directly enveloped by the muscimol infusion. Alterations in the level of learning-associated immediate early-gene expression in RSC are seen as a network effect following lesions of the hippocampal formation [23]. We suspect instead that enhanced inhibition targets the complexity of inhibitory circuitry in hippocampus[16] coupled to dynamic changes of parvalbumin-positive GABAergic inhibition associated with learning[19]. Our findings suggest that 'on-target' effects of muscimol are a more likely explanation.

From a physiological perspective, this interpretation requires that synaptic plasticity can sometimes occur in the absence of cell-firing. Indeed, this may be more

frequent than generally realised and has been considered since the original papers on LTP [33, 34]. The conceptually interesting idea is that a network may sometimes be able to change the pattern of synaptic weights on its input synapses 'secretly' from neurons further downstream. Our electrophysiology focused on the perforant path input to the dentate gyrus, but a similar 'silent learning' effect may occur on the entorhinal cortex layer III input to CA1 and on the entorhinal cortex layer II input to CA3; it would not necessarily require activation of recurrent circuitry in CA3. Understanding the detailed physiological mechanism was not the primary purpose of this initial study, but comments relevant to such a future project are appropriate. First, the complexities of dendritic inhibition in the hippocampus might allow for a failure of memory retrieval to be caused by a block of cell-firing due to muscimol activation of $GABA_A$ receptors expressed on the cell soma of hippocampal excitatory neurons innervated by parvalbumin-positive GABAergic interneurons[16, 17, 35]. An impact of $GABA_A$ receptors in the dendrites may, however, reflect a differential effect on tonic rather than phasic inhibition[36, 37], arising because low-dose muscimol acts preferentially (but not exclusively) at extra-synaptic $GABA_A$ sites mediating tonic inhibition. A modest increase in tonic inhibition may be permissive for postsynaptic backpropagating dendritic spikes[1, 2, 38]. A further possibility is that augmented $GABA_A$ mediated inhibition in dendrites may leave intrinsic changes in dendritic membrane potential unaffected, and these are now known to be permissive for place-cell formation[39] and behavioural time-scale synaptic plasticity[40]. Addressing these distinct possibilities will not be easy in freely-moving animals. The retrieval/encoding dissociation might be examined optogenetically or chemogenetically[41, 42] using appropriate promoter lines that would allow differential targeting of distinct GABAergic neurons. Interestingly, the possibility that changes in excitation-inhibition balance is relevant to unmasking latent memory has also recently been studied in humans[43].

To summarise, the phenomenon of ‘silent learning’ in the awake animal is compatible with dendritic computation and the complexity of inhibitory network connectivity in the hippocampus. It suggests that synaptic plasticity can lurk cryptically under conditions in which the network expression of new memory trace formation is prevented by somatic inhibition. It has not escaped our notice that such learning may

occur more often than is generally appreciated and, indeed, be a characteristic of several aspects of cognitive learning.

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AUTHOR CONTRIBUTIONS

RM conceived the study. JR conducted the behavioural studies, AM the electrophysiological studies, and MY the drug diffusion studies; LG, TT, SC and RM prepared the manuscript. We acknowledge the considerable contribution of three anonymous reviewers.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. A Novel Watermaze Protocol to Dissociate Encoding and Retrieval

(A) The experimental design with animal handling, drug-cannula implantation, DMP training for 8–10 sessions, followed by the series of 3 linked sessions for the drug infusions interspersed with an additional interleaved training.

(B) Aerial drawings of the watermaze across 3 linked sessions (repeated across sessions for different drug conditions), with exemplar daily locations of the single escape platform (black circles; see Figure S1). The location on a specific session 1 (s1; black continuous circle in NW quadrant) and that on the next session 2 (s2; black circle in SW quadrant) are each shown, with the dotted line in black reflecting where the platform was available after 60s, and the continuous line showing it being available (60–120 s; it was always hidden below the water). The black filled parts of the cartoons are the platforms (in proportion) and the surround black circle is the associated analysis zone (20 cm diameter, centred on each location, 4% of area of pool). The location used on s1 is shown for s2 as a red dotted line (the 'memory' location of s1, but not actually used on s2; likewise for s3 with respect to s2). Note the platform always stayed in the same location for all 4 trials, and was available immediately for escape on trials 2–4.

(C) Atlantis Platform that is unavailable for the first 60 s of trial 1 of each session, but then rises to near the water surface. Cartoons below (dotted and continuous line) are as in panel B.

(D) A series of 3 linked sessions when drug (or aCSF) is administered 30 min (CNQX) or 40 min (muscimol and D-AP5) before s2. The same locations are displayed as in (B). Note that the first 60 s of trial 1 of s2 offered the opportunity to retrieve the memory of s1 (red continuous line), while trials 1-4 of s2 are the opportunities for new memory encoding[of black continuous circle in SW (south-west) quadrant]. When encoding was blocked during s2 by a drug, preventing updating, s3 may have offered the opportunity to retrieve the location on s1 (red dotted line).

(E) Mean first-crossing latencies on trials 1-4 of multiple interleaved training sessions. These training sessions are interspersed between the successive 3 linked sessions (grey bars). Means ± SEM.

Figure 2. Impact and Time-Course of Distinct Drugs on Hippocampal Physiology *in vivo*

(A) Differential impact of 2 µl infusions of muscimol (0.38 nmoles, orange), D-AP5 (60 nmoles, pink) and CNQX (6 nmoles, blue) on the early-rising slope of evoked fEPSPs in the dentate gyrus to perforant path stimulation, normalised to the pre-drug baseline (n = 5 for all conditions). Arrow points to the drug infusion; yellow shading indicates when the behavioural test is applied (40–70 min later).

(B) Normalised averaged fEPSP values for baseline, end of test period (after test), and 3 hr post-infusion (end), normalization to aCSF baseline.

(C and D) Normalized changes in the population spike (PS).

(E) Impact of aCSF, muscimol and D-AP5 on the induction of LTP (yellow lightning marks), with the fEPSP slope re-normalised with respect to the pre-tetanus baseline (10 min) and compared to the corresponding drug-treated group that was not subject to tetanisation. A key new finding is intact LTP induction under low-dose muscimol as well as aCSF, but not D-AP5. Yellow shading reflects the daily timing and duration of the behavioural experiment. LTP data plotted for that time. For absolute PS data, see Figure S3.

(F) Averaged LTP data.

(G) Schematic of stimulating and recording electrodes, and drug cannula locations of rat brain *in vivo.* Detail in Figure S2. Paired two-tailed t test (versus chance): **p < 0.01, ****p < 0.0001. Means ± SEM.

Figure 3. Impact of Distinct Pharmacological Manipulations on Encoding and Retrieval, and on Memory Updating

(A) Illustrative paths of representative rats during trial 1 of 3 linked sessions for all 4 drug conditions. Black platform = location that session; continuous red line = location on previous session; dotted red line = platform location two sessions back; small green circle = start of swim path; small blue circle = end of swim-path; dotted blue line = latter part of the swim path, not calculated in the memory retrieval data, after the Atlantis Platform became available. The key finding of silent learning is shown for the representative muscimol treated animal. Note random search all over the watermaze on s2 (during the drug; middle), but focused search at the s2 location on s3 (right). With aCSF, the rat always searches at or very close to the previous session location; with D-AP5, searching

is optimal on s2 but at chance on s3; with CNQX, the rat fails to show either memory retrieval or new encoding.

(B) Impact of hippocampal drug infusion on encoding and retrieval. Quantitative measure of search in the zone around a platform location on each trial 1 of 3 linked sessions, with drug administered on s2 (drug conditions were counterbalanced and given blind). Search time is plotted for the platform location of the *preceding* session. Note stable above-chance performance for s1–s3 for aCSF condition (black bars), but a different pattern in each of the three drugs. Following the overall Drugs × Sessions interaction (see text), separate ANOVAs were conducted for each drug over s1–s3 (muscimol: $F_{1,45} = 4.49$, $p = 0.029$; D-AP5: $F_{1,45} = 4.53$, $p = 0.007$; CNQX: $F_{1,45} = 18.37$, $p = 0.001$). The key finding was chance performance in muscimol condition on s2 but recovery during retrieval on s3.

(C) Impact of hippocampal drug infusion on memory updating. Dissociable impact of the drugs on memory updating as measured on s3. In the aCSF and muscimol conditions, memory of s1 during s3 was at chance (successful updating) and significantly below that observed for D-AP5 and CNQX which are both above chance (no updating).

(D) Representative search paths on trial 1 of s3 reflecting updating (aCSF: path frequently crosses continuous red line of s2) or no updating (CNQX: path frequently crossed the dotted red line of s1). Paired two-tailed t test (versus chance): **$p < 0.01$. 4% chance = ratio of surface areas of search zone and pool area. Means ± SEM.

STAR♣METHODS

Silent learning

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Corresponding Author, Richard Morris (r.g.m.morris@ed.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rats

All experiments were performed on adult male Lister Hooded Rats (200–300 g on arrival; Charles River, UK) in accordance with the United Kingdom Home Office Animal Procedures Act (1986) conducted under a Project Licence (PPL 60/4566) held by RGM Morris. They were kept in a vivarium in the same building on a 12 hr lights on/off schedule, in group cages of 4 rats per cage, with free access to food and water.

METHOD DETAILS

Behavioural apparatus and the Atlantis Platform

The watermaze is an apparatus in which a variety of distinct behavioural protocols can be trained [44]. It consists of a large pool of water (2 m in diameter) from which rodents learn to escape onto a hidden platform whose top surface is 1.5 cm beneath the water surface (Supplementary Figure S1A). Latex solution is added to render the water cloudy and thereby hide the escape platform. Water temperature is regulated at 25°C such that escape is desirable, but the procedure is not stressful as this is only 12–13°C lower than body temperature.

While many studies have deployed a reference memory procedure in which the escape platform remains in the same location across days, an alternative is the so-called 'delayed matching to place' (DMP) protocol [7] in which the escape platform moves from one location to another. This 'everyday memory' protocol, with repeated training across days and weeks, requires the integrity of the septal (dorsal) and/or intermediate hippocampus[45]. Lesions placed at different positions along the longitudinal axis damaging up to 90%+ or less than 20%+ of the hippocampus have indicated that learning is mediated via dorsal and intermediate hippocampus, with intact ventral hippocampal tissue being insufficient for effective day-to-day learning. A key analytic feature of the DMP protocol is that it allows a clean dissociation between memory encoding and memory retrieval. Performance is typically characterised by a long escape latency on trial 1 as the animal searches for the platform whose location that day is still unknown, searching initially at the previous session's location, followed by rapid memory encoding during the 30 s period out of the water followed by relatively direct paths to that day's location on trials 2–4. Four trials per day are used to ensure that an effective memory is

formed of the daily location that can be recalled during trial 1 of the next session. The intervals between trials can be varied systematically, with the trial 1 to trial 2 interval being 20 min in this study, while trial 2 to trial 3, and trial 3 to trial 4 was kept short at no more than 5 s after the 30 s period on the platform. In this way also, the rapidly learned strategy of learning where to go in each session (one session per day) is maintained, even in the face of interventions such as the application of drugs. A large number of different platform locations can be used across sessions, some on an outer virtual ring and the others on the inner ring (Supplementary Figure S1B). This distribution of possible locations (n=24 in this study) encourages widespread searching.

Memory retrieval is displayed as preferential searching on trial 1 of each session in the location that the escape platform had occupied during the *previous* session. This was quantified by running trial 1 of each session as a rewarded probe test, using an 'on-demand' or 'Atlantis' platform [8, 46] (Supplementary Figure S1C) which remained inaccessible for 60 s. This consists of a 12 cm diameter escape platform mounted on a stainless-steel spindle, initially at the bottom of the pool and thus unavailable, but which can be computer controlled to rise to within 1.5 cm of the water surface on-demand. By making this platform unavailable for 60 s on trial 1, the trial serves as a memory 'probe' for the previous session; the platform then rises, the animals find it and then climb onto it in the usual way.

An overhead camera, associated DVD recorder and on-line analysis software co-developed by Watermaze Software (Edinburgh) and Actimetrics (Evanston, USA) are used to monitor the path taken, and measure latency, path-length etc. During the initial period of 60 s of trial 1, two separate measures of performance were computed:

- ***First-crossing latency:*** is the time in seconds until the animal first crosses the correct location (12 cm diameter) where the platform will become available. As the platform is not actually available until 60 s has passed, this is not strictly-speaking an 'escape latency' (the animal keeps swimming), but it serves as a 'surrogate' of escape latency. This measure was also computed for all 4 trials of the session.
- ***Memory search tendency:*** The second measure is computed from the time spent swimming in a virtual zone of 40 cm diameter centred on the location of the platform during the previous session. This time is normalised with respect to the full 60 s of the

trial, and represented as a percentage relative to the 4% area of the pool that the zone occupies. This 4% level represents 'chance' if the animal were to be swimming around the pool randomly.

This DMP task offers analytic advantages over the reference memory protocol. First, the learning of several different platform locations can be studied. Second, once the animals have received a number of initial training sessions (8–10 sessions), memory encoding and storage takes place in one session. Third, reversible interventions can be introduced (such as intrahippocampal drug infusions, conducted blind) using a within-subjects, repeated measures design – with all the associated advantages of both reduced variability and reduced use of animals.

Stereotactic surgery

Anaesthesia was induced using isoflurane (induction, 5%; maintenance, 1–2%; air-flow, 1 l/min) (Zoetis, USA). The animals were placed in the stereotactic frame (David Kopf Instruments, USA). Infusion guide cannulae (26 gauge, 4.4 mm length, C315, Plastics One, USA) with stylets (33 gauge) that protruded 0.5 mm below the end of the cannula were inserted into the hippocampus bilaterally through small holes drilled into the skull. Cannula implantation coordinate for the hippocampus is as follows: anterior-posterior (AP) from bregma, –4.00 mm; mediolateral (ML), ±3.00 mm; and dorsal-ventral (DV) from the dura, –2.66 mm (Paxinos G, Watson C (2007) The Rat Brain in Stereotaxic Coordinates, Ed 6. Amsterdam: Academic Press/Elsevier). Carprofen (0.08 ml/kg body weight; Zoetis) was administered by subcutaneous injection at the end of all surgical procedures. Animals recovered on a heating pad until normal behaviour resumed. All rats were allowed a recovery period of at least 7 days for them to regain their pre-surgery weights before behavioural testing.

Drugs

With respect to the drugs, phosphate-buffered artificial cerebrospmal fluid (aCSF) (in mM: 150 Na^+, 3 K^+, 1.4 Ca^{2+}, 0.8 Mg^{2+}, 155 Cl^-, 0.2 $H_2PO_4^-$, 0.8 HPO_4^{2-}, pH7.2) was used as the infusion vehicle and for control infusions. Drug concentrations for infusions were:

- **0.19 mM of the $GABA_A$ receptor agonist muscimol ($C_4H_6N_2O_2$; Tocris, UK)**

- **30 mM of the competitive NMDA receptor antagonist D-AP5 ($C_5H_{12}NO_5P$; Tocris)**
- **3 mM of the competitive AMPA/kainate receptor antagonist CNQX (disodium salt; $C_9H_2N_4O_4Na_2.H_2O$; Tocris)**

The pH of the drug solutions was adjusted to 7.2 by addition of 1 M NaOH solution (for D-AP5), or of concentrated phosphoric acid (for CNQX). Both aCSF and drug solutions were prepared in larger quantities, divided into small aliquots, and kept frozen at –20°C until they used. We facilitated the solution of CNQX by sonification. Note higher concentrations of muscimol were used in pilot studies.

Behavioural training

We used male Lister Hooded rats (n = 16, 250+ g) for the behavioural aspect of this study. They were stereotaxically implanted with bilateral guide cannulae targeting the dentate gyrus in the dorsal hippocampus. After recovery from surgery, they were trained on the DMP task over 8–10 sessions whereupon they showed the usual striking decline in escape latency between trials 1 and 2 of a session (Data S1) and above chance memory of the previous session's location during trial 1.

Thereafter, using a counterbalanced Latin-Square design, we examined the impact of aCSF, muscimol, D-AP5 and CNQX on performance in the DMP task. The 16 animals were all used as their own controls (i.e. every animal received each drug condition on different sessions) with ¼ of the group (i.e. 4 animals) being subject to any one drug on each drug session.

For microinfusion of drugs, the rats were habituated to the experimental procedure of injection for several days before the drug sessions in order to minimise stress. The stylets in the guide cannulae were replaced by two single infusion cannulae (33 gauge, Plastics One) connected to two 10 µl microsyringes (Hamilton, USA) in a microinfusion pump (World Precision, USA) via flexible plastic tubing filled with Fluorinert (3M, USA). The tips of infusion cannulae projected 0.5 mm below the tip of the guide cannulae. The drugs were bilaterally infused, in a volume of 2 µl per hemisphere, over a 4 min period with a 2 min period after drug infusion before the infusion cannulae were replaced with stylets. Rats received drug injection 30 min in the case of CNQX and 40 min in the case of

muscimol and D-AP5 prior to the start of training, and both 30 and 40 min (for different animals) for aCSF – this interval was based on data from *in vivo* electrophysiological recordings.

Drug sessions only occurred every 4th session as the test sessions were conducted as follows (Figure 1A). First, each set of sessions consisted of three successive sessions (called 3 linked sessions) that are referred to as session 1, session 2 and session 3 (s1, s2 and s3, respectively). The nomenclature is potentially confusing, because these 3 linked sessions were then repeated according to requirements of the Latin-Square design. Each repetitive 3 linked sessions always consisted of s1, s2 and s3 and the resulting scores were concatenated in Excel file until we had tested all 16 animals in each condition.

Second, in each 3 linked sessions, the drugs were only administered on s2. In this way, we could examine the following:

- **Impact of the presence of a drug (within the hippocampus) on memory retrieval of the location of the platform on the previous session (i.e. memory for s1).**
- **Impact of the drug on new learning (i.e. delivered during s2, but measured on s3 in the then drug-free state).**
- **Impact of the drug on 'updating' by comparing, on s3, the relative memory for s2 (the immediately preceding session) and that of s1 (two sessions before that).**

Third, these 3 linked sessions were separated by 1+ interleaved training session. The object of these additional training sessions was to maintain stability of the strategy of memory retrieval followed by new encoding, this being monitored by checking that the first-crossing latency remained stable throughout the experiment (which it did). The data plotted in Figure 1E shows first crossing latencies across the 4 trials of each interleaved training session; the longest time was spent searching on trial 1 with rapid escape to the newly learned location on trials 2–4. Representative search paths on critical probe trials are shown in Figure 3A, with the group behavioural data for the critical probe sessions shown in Data S3.

In earlier pilot work (behavioural and electrophysiological), we examined the impact of higher concentrations of muscimol (0.5 mM and 1.3 mM) and different time-periods after infusion for testing. In these cases, encoding during s2 and long-term potentiation (LTP) induction were each impaired. The dissociation between impaired retrieval and intact encoding only emerges at the low-dose of muscimol (0.19 mM).

Main Electrophysiology Studies

The aim of the electrophysiology was to provide data to guide the choice of drug doses for muscimol, D-AP5 and CNQX, and to examine excitatory synaptic transmission, cell-firing and LTP induction. We sought concentrations that definitively blocked LTP induction with D-AP5, but paradoxically spared LTP induction with muscimol.

Separate animals (male Lister-Hooded rats, weighing 250+ g, n=5 per drug condition) were used in the non-recovery electrophysiology studies. These animals were prepared for acute surgery in a stereotaxic apparatus (David Kopf Instruments) under non-recovery urethane anaesthesia (1.3 g/kg body weight; Sigma-Aldrich, USA), with the first intraperitoneal injection given during brief isofluorane anaesthesia (4% isoflurane in 0.8 l/min O_2). The electrophysiology studies typically lasted 6–8 hr, with the initial 2 hr being spent securing accurate placement of the stimulating and recording electrodes and cannula, and the subsequent 4 hr monitoring field-potential baseline and the impact of intrahippocampal drug infusions.

Stimulating and recording electrode positions are shown in Figure S2A. The stimulating electrode was a twisted bipolar Teflon-coated platinum-iridium electrode (20 μm diameter, 400 μm coated diameter for each of the two single strands) aimed at the angular bundle of the perforant path (AP 0.0 mm from lambda; ML 4.2 mm; DV 2.15 mm from dura). The recording electrode was a single Teflon coated platinum-iridium wire targeting the hilus of the dentate gyrus (AP 4.08 mm from bregma; ML 2.5 mm; DV 3.5 mm). The drug cannula was a 28 gauge stainless steel tube whose tip was, for the data reported in Figure 2, stereotaxically located at least 0.5 to 1.0 mm (± 0.3) mm away from the recording electrode (AP 3.6 mm from bregma; ML 2.6 mm; DV 3.5 mm).

Conventional field-potential recordings were made, with stimulation every 20 s, and these monitored and calculated on-line using EPS software [47]. In response to biphasic 200 μs stimulus pulses of circa 600–800 μA, we measured both the early-rising slope of the evoked potential by linear regression over several points, and the amplitude of the evoked dentate population spike. The stimulus intensity was adjusted to secure initial population spike amplitudes of circa 3–6 mV. LTP induction was attempted in some studies using a high-frequency stimulation protocol. This tetanic stimulation consisted of trains of 50 pulses (200 Hz), and repeated 3 times at an interval of 5 min [47].

Once acquired using suitable electrode placements, potentials typically remained relatively stable over periods of up to 3–4 hr, with a small upward drift of the population spike (but not fEPSP) that rarely exceeded 15% over this long period. Animals for which the potentials were unstable were discarded. The same long time-period stability was observed when aCSF was infused into the dorsal hippocampal formation at a depth targeting a region encompassing the stratum moleculare of area CA1. A volume of 2 μl was infused (0.5 μl/min) that, on the basis of previous autoradiographic data [24, 48] would be expected to diffuse throughout the entire CA1, CA3 and dentate gyrus regions of the septal (dorsal) hippocampus.

We then examined the impact of varying doses of drugs. We examined muscimol, D-AP5 and CNQX. Intrahippocampal infusions (2 μl) of artificial cerebrospinal fluid (aCSF, as vehicle), muscimol (0.19 mM), D-AP5 (30 mM) or CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 3 mM) were made into the dorsal hippocampus of male, Lister-hooded rats (n = 20, n = 5 per drug condition, aCSF at pH7.2; experimenter blind to drug administered; urethane anaesthesia; perforant path stimulation, recording electrode in the hilus of the dentate gyrus, drug cannula 1.0 mm distance, Figures 2G and S2A; Data S2). The infusion volume and doses were varied in pilot work, settling on a protocol of 2 μl for all three drugs that would, on the basis of autoradiographic data[13, 15, 48], likely affect the entire dorsal (septal) hippocampal formation, and extend to the intermediate region (to minimise the chances of a false negative in the behavioural study[45, 49]). While 2 μl is high, such a volume should still display minimal spread beyond hippocampus. The aim was to achieve:

- **Shutting down cell-firing but with minimal effect on fast synaptic transmission and LTP induction (with low-dose muscimol – 0.38 nanomoles)**
- **Blocking the induction of LTP with minimal effect on fast synaptic transmission or cell-firing (D-AP5 – 60 nanomoles).**
- **Shutting down both fast synaptic transmission and cell-firing (CNQX – 6 nanomoles)**

Effective doses for D-AP5 and CNQX were known from previous work[9, 10], but checked to realize internal consistency. Concentrations of 1.3, 0.5 and eventually 0.19 mM of muscimol were examined until, at the lowest dose, the dissociation we were seeking was secured with an infusion volume of 2 µl. Figure 2 shows the primary results (lowest dose of muscimol – 0.38 nanomoles).

Diffusion of muscimol

Critically, we sought to measure the diffusion of low-dose of muscimol along the longitudinal axis of the hippocampal formation in two ways: electrophysiology and fluorescent imaging. As outlined in the main text, we wondered if "silent learning" in the presence of muscimol could be an artefact of minimal drug diffusion from the site of dorsal infusion to the intermediate region of the hippocampal formation. This could leave intact hippocampal tissue to mediate learning. Accordingly, further electrophysiological experiments were conducted with the same low-dose of muscimol (0.38 nanomoles), but with the recording electrode location in the intermediate hippocampus (AP, −5.52 mm; ML, 3.8 mm; DV; 4.1 mm) - a distance of 2.0 to 2.5 mm from the infusion cannula (Figure S2B). Drug concentration and volume remained unchanged. Note that cell-firing remained substantially inhibited in this intermediate zone of the hippocampus (Figures S2C–S2E).

While these data reflect the impact of the drug, we also sought direct evidence of drug diffusion. This is tricky to do with some studies conducting radiography using tritiated (C^{14}) drugs, others using fluorescently labelled compounds. Muscimol is available as a fluorescently labelled compound that can be visualised microscopically, but it suffers from the difficulty that the molecular weight is much higher (607 instead of 114) and may be more "sticky" with respect to diffusion in aCSF. Accordingly, it is likely a conservative

estimate of diffusion but is at least a direct measure. Fluorescently-labeled muscimol-bodipy dissolved in aCSF (0.19 mM; Hello Bio, UK) was used to analyze the distribution profile of fluorescent muscimol 40 min after its bilaterally infusion into the dorsal hippocampus. Cannulae positions and the drug infusion procedure were same as in the main behavioural experiment. Brains were removed and shock-frozen on powdered dry ice, and 50-µm-thick coronal sections were acquired with a cryostat (CM1900, Leica Biosystems, Germany), and mounted on Silane-coated glass slides. Bright field and fluorescent images of serial sections equally spaced 100 µm were taken with a BZ-X700 Microscope (Keyence, Japan). Fluorescent images in grayscale were shown as arbitrarily assigned color display mode (pseudocolor) according to their gray levels within a range of 0–90[arbitrary units (AU)] and a 3D image showing the distribution of fluorescent signal was reconstructed from serial sections using a Metamorph software (Molecular Devices, USA). Overlaid images of bright-field and pseudocolor images were made using a Photoshop (Adobe, USA).

The problem of population spike potentiation

A separate complication with low-dose muscimol was that not only was it permissive for the induction of LTP measured using the early rising slope of the fEPSP, it was also permissive for spike potentiation. Thus, while minimal cell-firing was observed *before* LTP, some cell-firing under low-dose muscimol was observed *after* LTP induction (Figure S3). This was modest and so, rather than plot normalised data to a near-zero pre-tetanus baseline, absolute data are plotted. This raises the possibility that, in the behavioural study, it may have been possible for the animals to retrieve information about a previous session (s1) under low-dose musicimol after they had started encoding new information about platform location in the current session (s2) (because cell-firing might then be possible). In practice, we suspect such cell-firing is very unlikely in the behavioural situation. This is because LTP induction using 3 trains of 50 pulses at 250 Hz is an artificial tetanisation protocol designed to investigate activity-dependent synaptic plasticity *in vivo* but a firing pattern that does not occur during normal behavioural learning. Activity-dependent synaptic potentiation *in vivo* depends on more subtle patterns of neuronal activation, such as spike-timing dependent plasticity in a subset of neurons which is unlikely to cause much post-LTP cell-firing.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data Analysis

Behavioural Data Analysis

The measures of performance used were (1) first crossing latency (s) during training and (2) percent time spent swimming in a target zone during DMP probe tests. The retrieval tests were always on the first trial of each session and, as described above, involved the hidden platform being raised to within 1.5 cm of the water surface only after 60 s had passed.

Repeated-measures analysis of variance (ANOVA) was used to examine the impact of within subjects variables on behavioral measures with within subject factors session and condition. Orthogonal comparisons were used to further examine main effects of the ANOVA. Two-tailed one-sample t tests were used to compare search preference measures to the value expected by chance (4%). The level of significance was set $P < 0.05$. Data are presented as mean ± SEM. The statistical analysis was made using IBM SPSS Statistics (IBM, USA).

Electrophysiological Data Analysis

The analyses were done using routines implemented in Spike2, version 6.03 (Cambridge Electronic Design, UK). Quantitative measurements reflecting the fEPSP and the population spike (PS) activity were done following standard criteria. The PS recorded in the Dentate Gyrus is measured as the difference between the maximal negativity of the spike and the maximal point of the precedent positive crest. The fEPSP is measured as the steepest slope in the negative going potential in mV/ms.

All statistics were performed and plotted using GraphPad Prism 5.04 software (GraphPad Software, USA). For any statistical analysis shown, two-tailed repeated-measures t test (for two groups, or one group vs. a fixed number) or 1-way ANOVA (for more than two groups) for a significance level of $P < 0.05$ was used. When 2 factors concur, 2-way ANOVA is utilized with the same significance threshold. For repeated measures experiments, repeated-measures ANOVA are utilized. Post-hoc Bonferroni multiple-comparisons test is used to describe the origin of significance. All graphs represent Mean ± SEM.

Histological Data Analysis

For analysis of diffusion for fluorescent muscimol, areas in the dorsal/intermediate hippocampus, retrosplenial cortex and parietal association/secondary visual cortices were equally divided into grid squares (200 × 200 μm) and the averaged fluorescent intensity was measured. Also, we divided the entire cortical area at the injection point (i.e., –4.00 mm from the Bregma) into ten equal regions, calculated the averaged fluorescent intensity, and set the threshold as the mean + 2SD. The measurements of fluorescent intensity and area was made using a MetaMorph software.

DATA AND SOFTWARE AVAILABILITY

Electrophysiological and behavioural data are available upon request by contacting the Corresponding Authors, Richard Morris (r.g.m.morris@ed.ac.uk) and on-line.

SUPPLEMENTAL INFORMATION

Data S1: First-crossing latency for behavioural task during initial training displaying within day learning to approach the correct escape location across each daily set of 4 trials. Related to Figure 1E.

Data S2: Full electrophysiology data for Figure 2.

Data S3: Probe test data showing patterns of recall and new learning across different drug conditions.

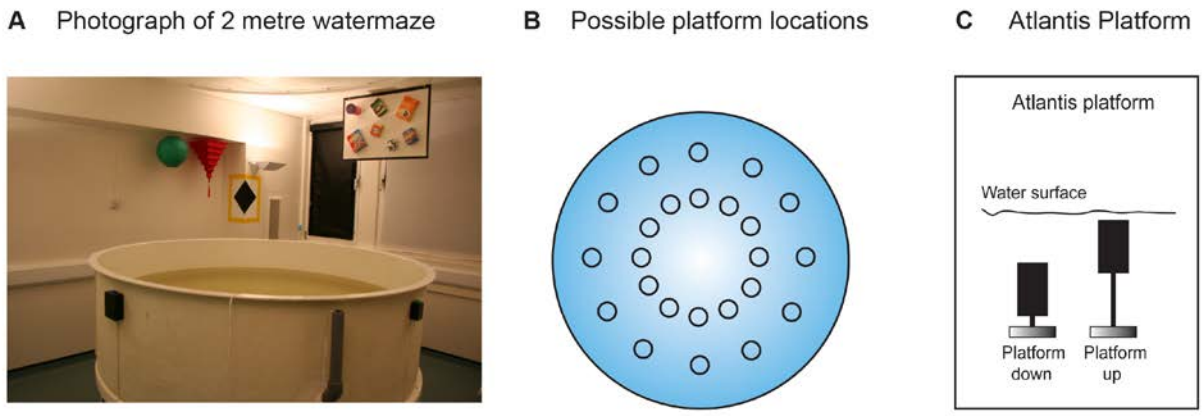

Figure S1. The Watermaze and Atlantis Platform. Related to Figure 1.
(A) Photograph of the pool and associated 3D extramaze cues in the laboratory in Edinburgh. (B) The possible platform locations (n=24), with only one used per session, located on virtual outer and inner rings as viewed by the overhead camera. (C) The 'on-demand' or 'Atlantis Platform' is unavailable at the bottom of the pool until it rises on a spindle until its top surface is 1.5 cm below the water surface, and thus available for escape.

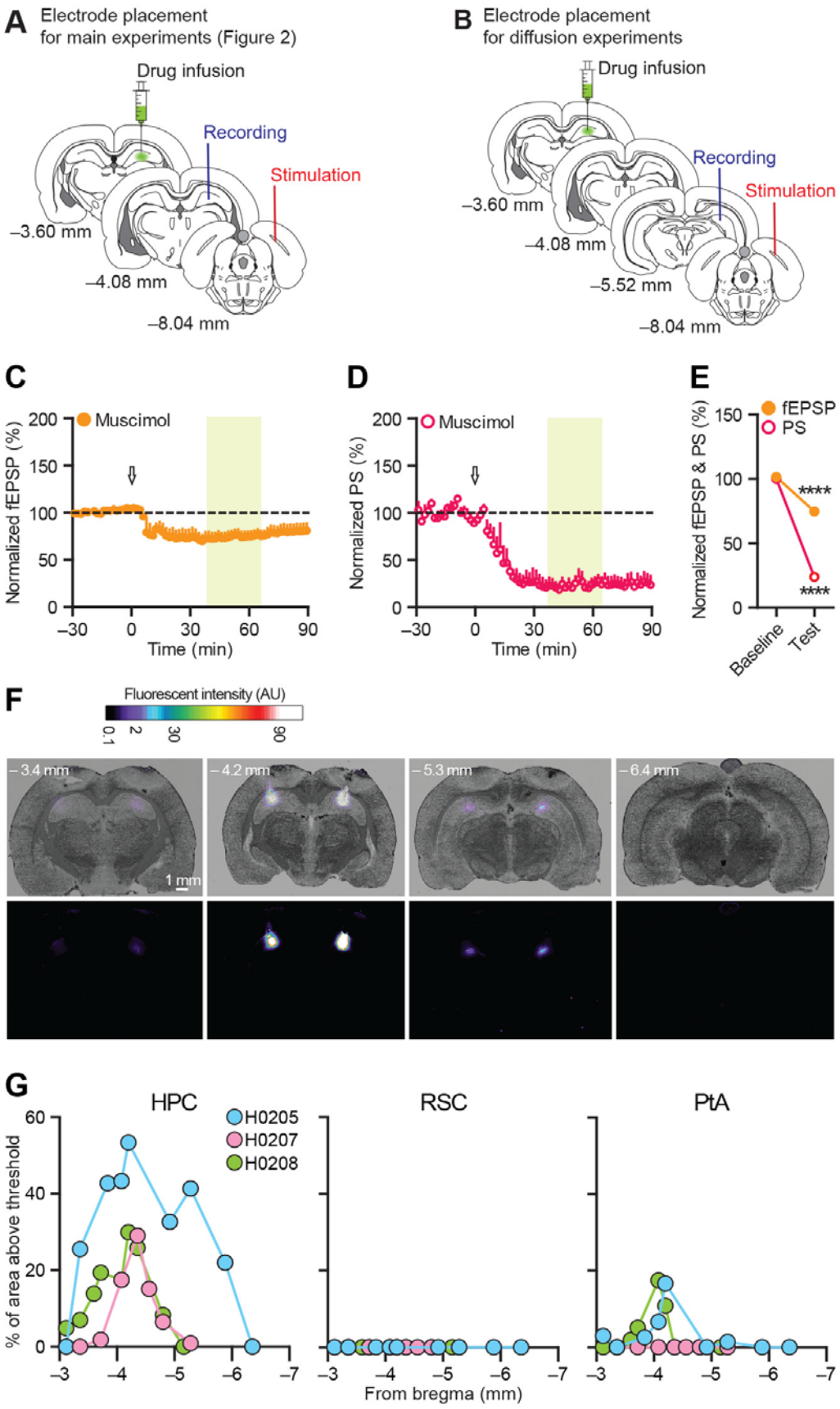

Figure S2. Electrophysiological analysis of effect drug diffusion through longitudinal axis of hippocampus, and of diffusion of a fluorescent labelled muscimol. Related to Figure 2. (A) Electrode

placements for the main study in which the recording electrode was within 0.5 mm from the AP location of the 2 ul drug infusion (i.e. region of maximal effect). Data shown in main Figure 2. (B) Electrode placements for the subsidiary study in which the recording electrode was 2 mm from the AP location of the 2 ul drug infusion. (C, D, E) fEPSP, Population Spike and averaged data (n=5) of the animals in the subsidiary study. The drug continues to have a substantial impact at 2 mm distance, suggesting a likely spread over 4 mm in the AP direction of muscimol. (F, G) Image and quantitation of diffusion of fluorescent muscimol bodipy from an infusion site at AP 4.0. One animal (H0205) showed diffusion of muscimol bodipy over approximately 3.0 mm, the other 2 animals showed less diffusion. There is no diffusion in the retrosplenial cortex, but some is detected in the parietal area immediately above the tip of infusion cannula reflecting "leakage" up the cannula track.

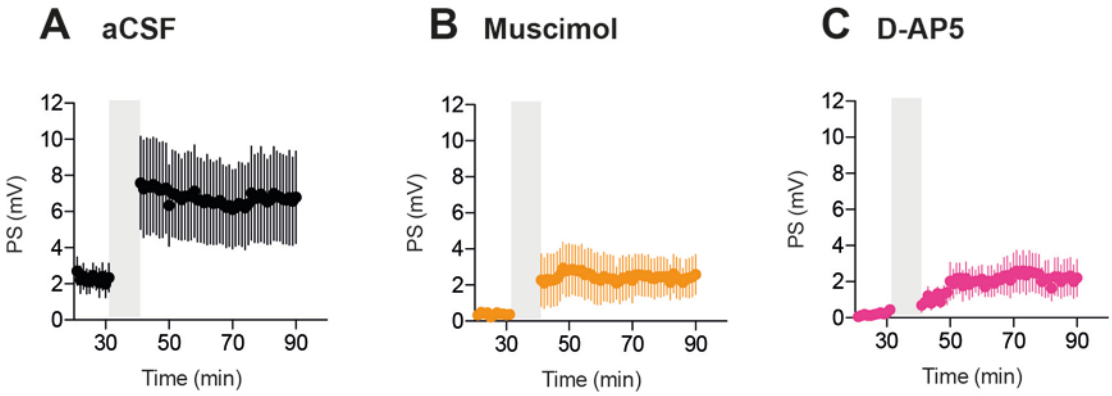

Figure S3. Population spike LTP shown in mV. The usual way in which LTP data is plotted is normalised to a pre-tetanus baseline. However, this is not sensible with a near zero baseline for the muscimol treated animals. Related to Figure 2.

(A) Clear potentiation of the population spike (PS) occurred after aCSF infusion, with the mean population spike reaching circa 8 mV. (B) Potentiation of the spike also occurred with low-dose muscimol (0.38 nanomoles), but the absolute magnitude was very small and a mean pop spike of circa 2 mV. (C) D-AP5 blocked spike potentiation, when judged against the steadily rising control condition (see Figure 2). However, some spiking is observed as with muscimol.